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COMPLETE SPECIFICATION

Title of Invention:

Novel dna and process for producing protein using the dna

Name, address and nationality of applicant(s) as in international application form:
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DESCRIPTION

NOVEL DNA AND PROCESS FOR PREPARING PROTEIN USING THE DNA

FIELD OF TECHNOLOGY

The present invention relates to a novel DNA and a process for preparing a protein which possesses an activity to inhibit osteoclast differentiation and/or maturation (hereinafter called osteoclastogenesis-inhibitory activity) by a genetic engineering technique using the DNA. More particularly, the present invention relates to a genomic DNA encoding a protein OCIF which possesses an osteoclastogenesis-inhibitory activity and a process for preparing said protein by a genetic engineering technique using the genomic DNA.

BACKGROUND OF THE INVENTION

Human bones are constantly repeating a process of resorption and formation. Osteoblasts controlling formation of bones and osteoclasts controlling resorption of bones take major roles in this process. Osteoporosis is a typical disease caused by abnormal metabolism of bones. This disease is caused when bone resorption by osteoclasts exceeds bone formation by osteoblasts. Although the mechanism of this disease is still to be elucidated completely, the disease causes the bones to ache, makes the bones fragile, and may result in fracturing of the bones. As the population of the aged increases, this disease results in an increase in the number of bedridden aged people which becomes a social problem. Urgent development of a therapeutic agent for this disease is strongly desired. Disease due to a

decrease in bone mass is expected to be treated by controlling bone resorption, accelerating bone formation, or improving balance between bone resorption and formation.

Osteogenesis is expected to be increased by accelerating proliferation, differentiation, or activation of the cells controlling the bone formation, or by controlling proliferation, differentiation, or activation of the cells involved in bone resorption. In recent years, strong interest has been directed to physiologically active proteins (cytokines) exhibiting such activities as described above, and energetic research is ongoing on this subject. The cytokines which have been reported to accelerate proliferation or differentiation of osteoblasts include the proteins of fibroblast growth factor family (FGF: Rodan S. B. et al., Endocrinology vol. 121, p 1917, 1987), insulin-like growth factor I (IGF-I: Hock J. M. et al., Endocrinology vol. 122, p 254, 1988), insulin growth factor II (IGF-II: McCarthy T. et al., Endocrinology vol. 124, p 301, 1989), Activin A (Centrella M. et al., Mol. Cell. Biol., vol. 11, p 250, 1991), transforming growth factor-β, (Noda M., The Bone, vol. 2, p 29, 1988), Vasculotropin (Varonique M. et al., Biochem. Biophys. Res. Commun., vol. 199, p 380, 1994), and the protein of heterotopic bone formation factor family (bone morphogenic protein; BMP: BMP-2; Yanaguchi A. et al., J. Cell Biol. vol. 113, p 682, 1991, OP-1; Sampath T. K. et al., J. Biol. Chem. vol. 267, p 20532. 1992, and Knutsen R. et al., Biochem. Biophys. Res. Commun. vol. 194, P 1352, 1993).

On the other hand, as the cytokines which suppress

differentiation and/or maturation of osteoclasts, transforming growth factor-β (Chenu C, et. al., Proc. Natl. Acad. Sci. USA, vol. 85, p 5683, 1988), interleukin-4 (Kasano K. et al., Bone-Miner., vol. 21, p 179, 1993), and the like have been reported. Further, as the cytokines which suppress bone resorption by osteoclast, calcitonin (Bone-Miner., vol. 17, p 347, 1992), macrophage colony stimulating factor (Hattersley G. et al., J. Cell. Physiol. vol. 137, p 199. 1988), interleukin-4 (Watanabe, K. et al., Biochem. Biophys. Res. Commun. vol. 172. P 1035, 1990), and interferon-γ (Gowen M. et al., J. Bone Miner. Res., vol. 1, p 46.9, 1986) have been reported.

These cytokines are expected to be used as agents for treating diseases accompanying bone loss by accelerating bone formation or suppressing bone resorption. Clinical tests are being undertaken to verify the effect of improving bone metabolism of some cytokines such as insulin-like growth

factor-I and the heterotopic bone formation factor family. In addition, calcitonin is already commercially available as a therapeutic agent for osteoporosis and a pain relief agent. At present, drugs for clinically treating bone diseases or shortening the period of treatment of bone diseases include activated vitamin D_3, calcitonin and its derivatives, and hormone preparations such as estradiol agent, ipriflavon or calcium preparations. These agents are not necessarily satisfactory in terms of the efficacy and therapeutic results. Development of a novel therapeutic agent which can be used in

M. et al. , Biochem. Biophys. Res. commun. vol.199, p380, 1994), and bone morphogenic protein (BMP:BMP-2; Yamaguchi, A et al., J. Cell Biol. vol. 113, p682, 1991, OP-1; Sampath T.K. et al., J. Biol. Chem. vol. 267, p20532, 1992, Knutsen R. et al., Biochem. Biophys. Res. Commun. vol. 194, p1352, 1992) were reported.

[0004]

On the other hand, as a cytokine inhibiting osteoclast formation, that is, differentiation and/or maturation of osteoclast, transforming growth factor-β (Chenu C. et al., Proc. Natl. Acad. Sci. USA, vol.85, p5683, 1988) and interleukin-4 (Kasano K. et al., Bone-Miner., vol.21, p179, 1993) were reported. And as a cytokine inhibiting bone resorption induced by osteoclast, calcitonin (Bone Miner., vol.17, p347, 1992), macrophage colony-stimulating factor (Hattersley G. et al., J. Cell. Physiol. vol.137, p199, 1988), interleukin-4 (Watanabe,K. et al., Biochem. Biophys. Res. Commun., vol.172, p1035, 1990) and interferon-τ (Gowen M. et al., J. Bone Miner. Res., vol.1, p469, 1986) were reported.

[0005]

These cytokines are expected to improve osteopenia by stimulating bone formation and inhibiting of bone resorption and clinical trial of some of the above-mentioned cytokines such as insulin like growth factor-I and cytokine of bone morphogenic protein family are being carried out as agents improving bone metabolism. Calcitonin has been already saled as a therapeutic agent for osteoporosis or a pain reliefing agent. In addition, for the treatment of metabolic bone diseases and for shortening treatment duration, active vitamin D_3, calcitonin and analogue

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thereof, hormones such as estradiol, ipriflavone or calcium agent etc. is clinically used at present. However, by these therapeutic methods, effects of the treatment is not necessarily satisfactory. Therefore, development of a novel therapeutic agent is desired in replace of the above methods.

[0006]

[Problems to be Solved by the Invention]

Considering these situations, the present inventors have eagerly studied and found that OCIF protein having an inhibitory activity on osteoclast formation was already recovered from culture medium of human embryonic lung fibroblast cell line IMR-90(ATCC deposit number is CCL186) (PCT/JP96/00374). Further, the present inventors have studied on the origin of OCIF having an inhibitory activity on osteoclast formation and determined base sequence of genomic DNA of human origined OCIF. An object of the present invention is to provide a genomic DNA encoding OCIF protein having an inhibitory activity on osteoclast formation and a method of preparing said protein by genetic engineering manipulation.

[0007]

[Means to Solve the Problem]

The present invention relates to a genomic DNA encoding protein OCIF having an inhibitory activity on osteoclast formation and a method of preparing said protein thereby by genetic engineering manipulation.

The DNA of the present invention comprises base sequence of

immunological diagnosis of such diseases. 33040

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a result of Western Blotting analysis of the protein obtained by causing genomic DNA of the present invention to express a protein in Example 4 (iii), wherein lane 1 indicates a size marker, lane 2 indicates the culture broth of COS7 cells in which a vector pWESRαOCIF (Example 4 (iii)) has been transfected, and lane 3 is the culture broth of COS7 cell in which a vector pWESRα(control) has been transfected.

BEST MODE FOR CARRYING OUT THE INVENTION

The genomic DNA encoding the protein OCIF which exhibits osteoclastogenesis-inhibitory activity in the present invention can be obtained by preparing a cosmid library using a human placenta genomic DNA and a cosmid vector and by screening this library using DNA fragments which are prepared based on the OCIF cDNA as a probe. The thus-obtained genomic DNA is inserted into a suitable expression vector to prepare an OCIF expression cosmid. A recombinant type OCIF can be obtained by transfecting the genomic DNA into a host organism such as various types of cells or microorganism strains and causing the DNA to express a protein by a conventional method. The resultant protein exhibiting osteoclastogenesis-inhibitory activity (an osteoclastogenesis-inhibitory factor) is useful as an agent for the treatment and improvement of diseases involving a decrease in bone mass such as osteoporosis and other diseases relating to bone metabolism abnormality and also as an antigen to prepare antibodies for establishing immunological diagnosis of such

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diseases. The protein of the present invention can be prepared as a drug composition for oral or non-oral administration. Specifically, the drug composition of the present invention containing the protein which is an osteoclastogenesis-inhibitory factor as an active ingredient can be safely administered to humans and animals. As the form of drug composition, a composition for injection, composition for intravenous drip, suppository, nasal agent, sublingual agent, percutaneous absorption agent, and the like are given. In the case of the composition for injection, such a composition is a mixture of a pharmacologically effective amount of the osteoclastogenesis-inhibitory factor of the present invention and a pharmaceutically acceptable carrier. The composition may further comprise amino acids, saccharides, cellulose derivatives, and other excipients and/or activation agents, including other organic compounds and inorganic compounds which are commonly added to a composition for injection. When an injection preparation is prepared using the osteoclastogenesis-inhibitory factor of the present invention and these excipients and activation agents, a pH adjuster, buffering agent, stabilizer, solubilizing agent, and the like may be added if necessary to prepare various types of injection agents.

The present invention will now be described in more detail by way of examples which are given for the purpose of illustration and not intended to be limiting of the present invention.

Example 1

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<Preparation of a cosmid library>

A cosmid library was prepared using human placenta genomic DNA (Clonetech; Cat. No. 6550-2) and pWE15 cosmid vector (Stratagene). The experiment was carried out following principally the protocol attached to the pWE15 cosmid vector kit of Stratagene Company, provided Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory (1989)) was referred to for common procedures for handling DNA, E. coli, and phage.

(i) Preparation of restriction enzyme digest of human-genomic DNA

Human placenta genomic DNA dissolved in 750 µl of a solution containing 10 mM Tris-HCl, 10 mM $MgCl_2$, and 100 mM NaCl was added to four 1.5 ml Eppendorf tubes (tube A, B, C, and D) in the amount of 100 µg each. Restriction enzyme MboI was added to these tubes in the amounts of 0.2 unit for tube A, 0.4 unit for tube B, 0.6 unit for tube C, and 0.8 unit for tube D, and DNA was digested for 1 hour. Then, EDTA in the amount to make a 20 mM concentration was added to each tube to terminate the reaction, followed by extraction with phenol/chloroform (1:1). A two-fold amount of ethanol was added to the aqueous layer to precipitate DNA. DNA was collected by centrifugation, washed with 70% ethanol, and DNA in each tube was dissolved in 100 µl of TE (10mM Tris-HCl (pH 8.0) + 1mM EDTA) buffer solution, hereinafter called TE). DNA in four tubes was combined in one tube and incubated for 10 minutes at 68°C. After cooling to room

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temperature, the mixture was overlayed onto a 10%-40% linear sucrose gradient which was prepared in a buffer containing 20 mM Tris-HCl (pH 8.0), 5mM EDTA, and 1M NaCl in a centrifuge

tube (38 ml). The tube was centrifuged at 26,000 rpm for 24 hours at 20℃ using a rotor SRP28SA manufactured by Hitachi, Ltd. and 0.4 ml fractions of the sucrose gradient was collected using a fraction collector. A portion of each fraction was subjected to 0.4% agarose electrophoresis to confirm the size of DNA. Fractions containing DNA with a length of 30 kb (kilo base pair) to 40 kb were thus combined. The DNA solution was diluted with TE to make a sucrose concentration to 10% or less and 2.5-fold volumes of ethanol was added to precipitate DNA. DNA was dissolved in TE and stored at 4℃.

(ii) Preparation of cosmid vector

The pWE15 cosmid vector obtained from Stratagene Company was completely digested with restriction enzyme BamHI according to the protocol attached to the cosmid vector kit. DNA collected by ethanol precipitation was dissolved in TE to a concentration of 1 mg/ml. Phosphoric acid at the 5'-end of this DNA was removed using calf small intestine alkaline phosphatase, and DNA was collected by phenol extraction and ethanol precipitation. The DNA was dissolved in TE to a concentration of 1 mg/ml.

(iii) Ligation of genomic DNA to vector and in vitro packaging

1.5 micrograms of genomic DNA fractionated according to size and 3 μg of pWE15 cosmid vector which was digested with

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restriction enzyme BamHI were ligated in 20 µl of a reaction solution using Ready-To-Go T4DNA ligase of Pharmacia Company. The ligated DNA was packaged in vitro using Gigapack™ II packaging extract (Stratagene) according to the protocol. After the packaging reaction, a portion of the reaction mixture was diluted stepwise with an SM buffer solution and mixed with E. coli XL1-Blue MR (Stratagene) which was suspended in 10 mM $MgCL_2$ to cause phage to infect, and plated onto LB agar plates containing 50 µg/ml of ampicillin. The number of colonies produced was counted. The number of colonies per 1 µl of packaging reaction was calculated based on this result.

(iv) Preparation of a cosmid library

The packaging reaction solution thus prepared was mixed with E. coli XL1-Blue MR and the mixture was plated onto agarose plates containing ampicillin so as to produce 50,000 colonies per agarose plate having a 15 cm of diameter. After incubating the plate overnight at 37°C, an LB culture medium was added in the amount of 3 ml per plate to suspend and collect E. coli cells. Each agarose plate was again washed with 3 ml of LB culture medium and the washing was combined with the original suspension of E. coli. The E. coli collected from all agarose plates was placed in a centrifuge tube, glycerol was added to a concentration of 20%, and ampicillin was further added to make a final concentration of 50µg/ml. A portion of the E. Coli suspension was removed and the remainder was stored at -80°C. The removed E. Coli was diluted stepwise and plated onto agar plates to estimate the number of colonies per 1 ml of

suspension.

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Example 2

<Screening of cosmid library and purification of colony>

A nitrocellulose filter (Millipore) with a diameter of 14.2 cm was placed on each LB agarose plate with a diameter of 15 cm which contained 50 μg/ml of ampicillin. The cosmid library was plated onto the plates so as to produce 50,000 colonies of E. coli per plate, followed by incubation overnight at 37℃. E. coli on the nitrocellulose filter was transferred to another nitrocellulose filter according to a conventional method to obtain two replica filters. According to the protocol attached to the cosmid vector kit, cosmid DNA in the E. coli on the replica filters was denatured with an alkali, neutralized, and immobilized on the nitrocellulose filter using a Stratalinker (Stratagene). The filters were heated for two hours at 80℃ in a vacuum oven. The nitrocellulose filters thus obtained were hybridized using two kinds of DNA produced, respectively, from 5'-end and 3'-end of human OCIF cDNA as probes. Namely, a plasmid was purified from E. coli pKB/OIF10 (deposited at The Ministry of International Trade and Industry, the Agency of Industrial Science and Technology, Biotechnology Laboratory, Deposition No. FERM BP-5267) containing OCIF cDNA. The plasmid containing OCIF cDNA was digested with restriction enzymes KpnI and EcoRI. Fragments thus obtained were separated using agarose gel electrophoresis. KpnI/EcoRI fragment with a length of 0.2 kb was purified using a QIAEX II gel extraction kit (Qiagen). This DNA was labelled with ^{32}P using the Megaprime DNA labelling

System (Amersham) (5'-DNA probe). Apart from this, a BamHI/EcoRV fragment with a length of 0.2 kb which was produced from the above plasmid by digestion with restriction enzymes BamHI and EcoRV was purified and labeled with ^{32}P (3'-DNA probe). One set of the replica filters described above was hybridized with the 5'-DNA probe and the other with the 3'-DNA probe. Hybridization and washing of the filters were carried out according to the protocol attached to the cosmid vector kit. Autoradiography detected several positive signals with each probe. One colony which gave positive signals with both probes was identified. The colony on the agar plate, which corresponding to the signal on the autoradiogram was isolated and purified. A cosmid was prepared from the purified colony by a conventional method. This cosmid was named pWEOCIF. The size of the human genomic DNA fragment contained in this cosmid was about 38 kb.

Example 3

<Determination of the nucleotide sequence of the human OCIF genomic DNA fragment>

(i) Subcloning of the OCIF genomic DNA fragment

Cosmid pWEOCIF was digested with restriction enzyme EcoRI. After the separation of the DNA fragments thus produced by electrophoresis using a 0.7% agarose gel, the DNA fragments were transferred to a nylon membrane (Hybond -N, Amasham) by the Southern blot technique and immobilized on the nylon membrane using Stratalinker (Stratagene). On the other hand, plasmid pBKOCIF was digested with restriction enzyme EcoRI and a 1.6

kb fragment containing human OCIF cDNA was isolated by agarose gel electrophoresis. The fragment was labeled with ^{32}P using the Megaprime DNA labeling system (Amasham).

Hybridization of the nylon membrane described above with the ^{32}P-Labelled 1.6=kb OCIF cDNA, performed according to a conventional method, detected DNA fragments with a size of 6 kb, 4 kb, 3.6 kb, and 2.6 kb. These fragments hybridized with the human OCIF cDNA were isolated using agarose gel electrophoresis and individually subcloned into an EcoRI site of pBluescript II SK + vector (Strategene) by a conventional method. The resulting plasmids were named respectively, pBSE 6, pBSE 4, pBSE 3.6, and PBSE 2.6.

(ii) Determination of the nucleotide sequence

The nucleotide sequences of the human OCIF genomic DNA fragments which were subcloned into the plasmid were determined using the ABI Dideoxy Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and the 373 Sequencing System (Applied Biosystems). The primers used for the determination of the nucleotide sequences were synthesized based on the nucleotide sequence of human OCIF cDNA (Sequence ID No. 4 in the Sequence Table). The nucleotide sequences thus determined are given as the Sequences No. 1 and No. 2 in the Sequence Table. The sequence ID No. 1 includes the first exon of the OCIF gene and the Sequence ID No. 2 includes the second, third, fourth, and fifth exons. A stretch of about 17 kb is present between the first and second exons.

Example 4

<Production of recombinant OCIF using COS-7 cells>

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(i) Preparation of OCIF genomic DNA expression cosmid

To express OCIF genomic DNA in animal cells, an expression unit of expression plasmid pcDL-SRα296 (Molecular and Cellar Biology, vol. 8, P466-472, 1988) was inserted into cosmid vector pWE15 (Stratagene). First of all, the expression plasmid pcDL-SRα296 was digested with a restriction enzyme Sal I to cut out expression unit with a length of about 1.7 kb which includes an SRα promotor, SV40 later splice signal, poly (A) addition signal, and so on. The digestion products were separated by agarose gel electrophoresis and the 1.7-kb fragment was purified using the QIAEX II gel extraction kit (Qiagen). On the other hand, cosmid vector pWE15 was digested with a restriction enzyme EcoRI and fragments were separated using agarose gel electrophoresis. pWE15 DNA of 8.2 kb long was purified using the QIAEX II gel extraction kit (Qiagen). The ends of these two DNA fragments were bluntled using a DNA blunting kit (Takara Shuzo), ligated using a DNA ligation kit (Takara Shuzo), and transfected into E. coli DH5α (Gibco BRL). The resultant transformant was grown and the expression cosmid pWESRα containing the expression unit was purified using a Qiagen column (Qiagen).

The cosmid pWE OCIF containing the OCIF genomic DNA fragment with a length of about 38 kb obtained in (i) above was digested with a restriction enzyme NotI to cut out the OCIF genomic DNA fragment of about 38 kb. After separation by agarose gel electrophoresis, the DNA fragment was purified using the QIAEX II gel extraction kit (Qiagen). On the other hand, the expression cosmid pWESRα was

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digested with a restriction enzyme EcoRI and the digestion product was extracted with phenol and chloroform, ethanol-precipitated, and dissolved in TE.

pWESRα digested with a restriction enzyme EcoRI and an EcoRI-XmnI-NotI adapter (#1105, #1156 New England Biolaboratory Co.) were ligated using T4 DNA ligase (Takara Shuzo Co., Ltd.). After removal of the free adapter by agarose gel electrophoresis, the product was purified using QIAEX gel extraction kit (Qiagen). The OCIF genomic DNA fragment with a length of about 37 kb, which was derived from the digestion with restriction enzyme NotI and the pWESRα to which the adapter was attached were ligated using T4 DNA ligase (Takara Shuzo). The DNA was packaged in vitro using the Gigapack packaging extract (Stratagene) and transfected to E. coli XL1-Blue MR (Stratagene). The resultant transformant was grown and the expression cosmid pWESRα OCIF which contained OCIF genomic DNA fragment was purified using a Qiagen column (Qiagen). The OCIF expression cosmid pWESRα OCIF was ethanol-precipitated, dissolved in sterile distilled water and used in the following analysis.

(ii) <u>Transient expression of OCIF genomic DNA and measurement of OCIF activity</u>

A recombinant OCIF was expressed as described below using the OCIF expression cosmid pWESRαOCIF obtained in (i) above and its activity was measured. COS-7 (8×10^5 cells/well) cells (Riken Cell Bank, RCB0539) were plated in a 6-well plate using DMEM culture medium (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL). On the following day, the culture

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medium was removed and cells were washed with serum-free DMEM culture medium. The OCIF expression cosmid pWESRαOCIF which had been diluted with OPTI-MEM culture medium (Gibco BRL) was mixed with lipophectamine and the mixture was added to the cells in each well according to the attached protocol. The expression cosmid pWESRα was added to the cells in the same manner as a control. The amount of the cosmid DNA and Lipophectamine was respectively 3 μg and 12 μl. After 24 hours, the culture medium was removed and 1.5 ml of fresh EX-CELL 301 culture medium (JRH Bioscience) was added to each well. The culture medium was recovered after 48 hours and used as a sample for the measurement of OCIF activity. The measurement of OCIF activity was carried out according to the method described by Kumegawa, M. et al. (Protein, Nucleic Acid, and Enzyme, Vol. 34, p 999 (1989)) and the method of TAKAHASHI, N. et al. (Endocrihology vol. 122, p 1373 (1988)). The osteoclast formation from bone marrow cells isolated from mice aged about 17 days in the presence of activated vitamin D_3 was evaluated by the induction of tartaric acid resistant acidic phosphatase activity. The reduction of the acid phosphatase was measured and used as the activity of the protein which possesses osteoclastogenesis-inhibitory activity (OCIF). Namely, 100 μl of a OCIF sample which was diluted with α-MEM culture medium (Gibco BRL) containing $2x10^{-8}$ M activated vitamin D_3 and 10% fetal bovine serum was added to each well of a 96 well micro plate. Then, $3x10^5$ bone marrow cells isolated from mice (about 17-days old) suspended in 100 μl of α-MEM culture medium containing 10% fetal bovine serum

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were added to each well of the 96 well micro plate and cultured for a week at 37℃ and 100% humidity under 5% CO_2 atmosphere. On days 3 and 5, 160 µl of the conditioned medium was removed from each well, and 160 µl of a sample which was diluted with α-MEM culture medium containing $1x10^{-8}$ M activated vitamin D_3 and 10% fetal bovine serum was added. On day 7, the cells were washed with a phosphate buffered saline and fixed with a ethanol/acetone (1:1) solution for one minute at room temperature. The osteoclast formation was detected by staining the cells using an acidic phosphatase activity measurement kit (Acid Phosphatase, Leucocyte, Cat. No. 387-A, Sigma Company). A decrease in the number of cells positive to acidic phosphatase activity in the presence of tartaric acid was taken as the OCIF activity. The results are shown in Table 1, which indicates that the conditioned medium exhibits the similar activity to natural type OCIF obtained from the IMR-90 culture medium and recombinant OCIF produced by CHO cells.

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TABLE 1

Activity of OCIF in the conditioned medium of COS-7 cells.

Dilution	1/10	1/20	1/40	1/80	1/160	1/320
OCIF genomic DNA introduced	++	++	++	++	+	-
Vector introduced	-	-	-	-	-	-
Untreated	-	-	-	-	-	-

"++" indicates an activity inhibiting 80% or more of osteoclast formation, "+" indicates an activity inhibiting 30-80% of osteoclast formation, and "-" indicates that no inhibition of osteoclast formation is observed.

(iii) Identification of the product by Western Blotting

A buffer solution (10 µl) for SDS-PAGE (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 20 µg/ml bromophenol blue, pH 6.8) was added to 10 µl of the sample for the measurement of OCIF activity prepared in (ii) above. After boiling for 3 minutes at 100℃, the mixture was subjected to 10% SDS polyacrylamide electrophoresis under non-reducing conditions. The proteins were transferred from the gel to a PVDF membrane (ProBlott, Perkin Elmer) using semi-dry blotting apparatus (Biorad). The membrane was blocked and incubated for 2 hours at 37℃ together with a horseradish peroxidase-labeled anti-OCIF antibody obtained by labelling the previously obtained anti-OCIF antibody with horseradish peroxidase according to a conventional method. After washing, the protein which bound the anti-OCIF antibody was detected using the ECL system (Amasham). As shown in Figure 1, two bands, one with a molecular weight of about 120 kilo dalton and the other 60 kilo dalton, were detected in

the supernatant obtained from the culture broth of COS-7 cells in which pWESRαOCIF was transfected. On the other hand, these two bands with a molecular weight of about 120 kilo dalton and 60 kilo dalton were not detected in the supernatant obtained from the culture broth of COS-7 cells in which pWESRα vector was transfected, confirming that the protein obtained was OCIF.

INDUSTRIAL APPLICABILITY

The present invention provides a genomic DNA encoding a protein OCIF which possesses an osteoclastogenesis-inhibitory activity and a process for preparing this protein by a genetic engineering technique using the genomic DNA. The protein obtained by expressing the gene of the present invention exhibits an osteoclastogenesis-inhibitory activity and is useful as an agent for the treatment and improvement of diseases involving a decrease in the amount of bone such as osteoporosis, other diseases resulting from bone metabolism abnormality such as rheumatism, degenerative joint disease, and multiple myeloma. The protein is further useful as an antigen to establish antibodies useful for an immunological diagnosis of such diseases.

NOTE ON MICROORGANISM

Depositing Organization:

The Ministry of International Trade and Industry, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology

Address: 1-3, Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan

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[0024]

Seq.Id.No.: 1

Length of sequence: 1316

Type of sequence: nucleic acid

Strandedness: 2

Topology: linear

Molecular type: genomic DNA(human OCIF genomic DNA-1)

Sequence:

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CTGGAGACAT ATAACTTGAA CACTTGGCCC TGATGGGGAA GCAGCTCTGC AGGGACTTTT   60
TCAGCCATCT GTAAACAATT TCAGTGGCAA CCCGCGAACT GTAATCCATG AATGGGACCA  120
CACTTTACAA GTCATCAAGT CTAACTTCTA GACCAGGGAA TTAATGGGGG AGACAGCGAA  180
CCCTAGAGCA AAGTGCCAAA CTTCTGTCGA TAGCTTGAGG CTAGTGGAAA GACCTCGAGG  240
AGGCTACTCC AGAAGTTCAG CGCGTAGGAA GCTCCGATAC CAATAGCCCT TTGATGATGG  300
TGGGGTTGGT GAAGGGAACA GTGCTCCGCA AGGTTATCCC TGCCCCAGGC AGTCCAATTT  360
TCACTCTGCA GATTCTCTCT GGCTCTAACT ACCCCAGATA ACAAGGAGTG AATGCAGAAT  420
AGCACGGGCT TTAGGGCCAA TCAGACATTA GTTAGAAAAA TTCCTACTAC ATGGTTTATG  480
TAAACTTGAA GATGAATGAT TGCGAACTCC CCGAAAAGGG CTCAGACAAT GCCATGCATA  540
AAGAGGGGCC CTGTAATTTG AGGTTTCAGA ACCCGAAGTG AAGGGGTCAG GCAGCCGGGT  600
ACGGCGGAAA CTCACAGCTT TCGCCCAGCG AGAGGACAAA GGTCTGGGAC ACACTCCAAC  660
TGCGTCCGGA TCTTGGCTGG ATCGGACTCT CAGGGTGGAG GAGACACAAG CACAGCAGCT  720
GCCCAGCGTG TGCCCAGCCC TCCCACCGCT GGTCCCGGCT GCCAGGAGGC TGGCCGCTGG  780
CGGGAAGGGG CCGGGAAACC TCAGAGCCCC GCGGAGACAG CAGCCGCCTT GTTCCTCAGC  840
CCGGTGGCTT TTTTTTCCCC TGCTCTCCCA GGGGACAGAC ACCACCGCCC CACCCCTCAC  900
GCCCCACCTC CCTGGGGGAT CCTTTCCGCC CCAGCCCTGA AAGCGTTAAT CCTGGAGCTT  960
TCTGCACACC CCCCGACCGC TCCCGCCCAA GCTTCCTAAA AAGAAAGGT GCAAAGTTTG 1020
GTCCAGGATA GAAAAATGAC TGATCAAAGG CAGGCGATAC TTCCTGTTGC CGGGACGCTA 1080
TATATAACGT GATGAGCGCA CGGGCTGCGG AGACGCACCG GAGCGCTCGC CCAGCCGCCG 1140
CCTCCAAGCC CCTGAGGTTT CCGGGGACCA CA ATG AAC AAG TTG CTG TGC TGC   1193
                                    Met Asn Lys Leu Leu Cys Cys
                                        -20                 -15
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GCG CTC GTG GTAAGTCCCT GGGCCAGCCG ACGGGTGCCC GGCGCCTGGG 1242
Ala Leu Val

GAGGCTGCTG CCACCTGGTC TCCCAACCTC CCAGCGGACC GGCGGGGAGA AGGCTCCACT 1302
CGCTCCCTCC CAGG 1316

[0025]

Seq.Id.No.: 2

Length of sequence: 9898

Type of sequence: nucleic acid

Strandedness: 2

Topology: linear

Molecular type: genomic DNA (human OCIF genomic DNA-2)

Sequence:

GCTTACTTTG TGCCAAATCT CATTAGGCTT AAGGTAATAC AGGACTTTGA GTCAAATGAT 60
ACTGTTGCAC ATAAGAACAA ACCTATTTTC ATGCTAAGAT GATGCCACTG TGTTCCTTTC 120
TCCTTCTAG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG TTT 171
Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr Phe
-10 -5 1

CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG TTG 219
Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu Leu
5 10 15

TGT GAC AAA TGT CCT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA GCA 267
Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Ala
20 25 30 35

AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA GAC 315

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Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr Asp
40 45 50

AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC AAG 363
Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys Lys
55 60 65

GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC GTG 411
Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val
70 75 80

TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG AAA 459
Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys
85 90 95

CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT G GTACGTGTCA 509
His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
100 105 110

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GCTAACAATA AGCAGTTATA ATTAATTATG TAAAAAATGA GAATGGTGAG GGGAATTGCA 989
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TGCCACATTT GCGAAGCTTC AGTGCAGCCT ATAACTTTTC ATAGCTTGAG AAAATTAAGA 1289
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CAGTGTTTCT CAACTGAAGC CCTGCTGATA TTTTAAGAAA TATCTGGATT CCTAGGCTGG 1409
ACTCCTTTTT GTGGGCACCT GTCCTGCGCA TTGTAGAATT TTGGCAGCAC CCCTGGACTC 1469
TAGCCACTAG ATACCAATAG CAGTCCTTCC CCCATGTGAC AGCCAAAAAT GTCTTCAGAC 1529
ACTGTCAAAT GTCGCCAGGT GGCAAAATCA CTCCTGGTTG AGAACAGGGT CATCAATGCT 1589
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CTATTGGATG GTACTTTGAG ACTCAAAAGC TAAGCTAAGT TGTGTGTGTG TCAGGGTGCG 1889
GGGTGTGGAA TCCCATCAGA TAAAAGCAAA TCCATGTAAT TCATTCAGTA AGTTGTATAT 1349
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CTAATGAAGT GAAAAATGAA AATGCTAGAG TTTTGTGCAA CATAATAGTA GCAGTAAAAA 4409
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GAGAAGGTAC TAAATTGCTT GGTATTTTCC GTAG GA ACC CCA GAG CGA AAT ACA 4523

Gly Thr Pro Glu Arg Asn Thr

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115

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GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA AAT GAG ACG TCA TCT   4571
Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser
120                 125                 130                 135

AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT GTC TTT GGT CTC CTG   4619
Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu
                140                 145                 150

CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC ATA TGT TCC GGA AAC   4667
Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn
            155                 160                 165

AGT GAA TCA ACT CAA AAA TGT GGA ATA G GTAATTACAT TCCAAAATAC       4715
Ser Glu Ser Thr Gln Lys Cys Gly Ile
        170                 175

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CGATGCATTA CTGGTAAAGC TACCACTCAG AATCTCTCAA AAACTCATCT TCTCACAGAT 4895
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CAGGAGATGG AGGTTGCAGT GAGCTGAGAT TGTACCACTG CACTCCAGTC TGGGCAACAG 5375
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180 185

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Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val
190 195 200

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GAC AAT TTG CCT GGC ACC AAA GTA AAC GCA GAG AGT GTA GAG AGG ATA 6843
Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
205 210 215

AAA CGG CAA CAC AGC TCA CAA GAA CAG ACT TTC CAG CTG CTG AAG TTA 6891
Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
220 225 230 235

TGG AAA CAT CAA AAC AAA GAC CAA GAT ATA GTC AAG AAG ATC ATC CAA G 6940
Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile Ile Gln
240 245 250

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Asp Ile Asp

CTC TGT GAA AAC AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC 8724
Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr
255 260 265 270

TTC GAG CAG CTT CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG 8772
Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val
275 280 285

GGA GCA GAA GAC ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC 8820
Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp
290 295 300

CAG ATC CTG AAG CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA 8868
Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln

305 310 315

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Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr
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CAC TTT CCC AAA ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC 8964
His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe
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370 375 380

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ATTT 9898

[0026]

Seq.Id.No.: 3

Length of sequence: 401

Type of sequence: amino acid

Strandedness: 1

Topology: linear

Molecular type: protein

Sequence:

Met Asn Asn Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser
-20 -15 -10
Ile Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His
-5 1 5
Tyr Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro
10 15 20
Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr
25 30 35
Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His
40 45 50
Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu
55 60 65
Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val Cys
70 75 80
Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys
85 90 95
His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr

100 105 110

Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe

115 120 125

Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn

130 135 140

Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr

145 150 155

His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys

160 165 170

Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala

175 180 185

Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp

190 195 200

Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile

205 210 215

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys

220 225 230

Leu Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile

235 240 245

Ile Gln Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile

250 255 260

Gly His Ala Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu

265 270 275

Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr

280 285 290

Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser

295 300 305

Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu

310 315 320

Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys Thr
325 330 335

Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe
340 345 350

Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly
355 360 365

Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
370 375 380

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[0027]

Seq.Id.No.: 4

Length of sequence: 1206

Type of sequence: nucleic acid

Strandedness: 1

Topology: linear

Molecular type: cDNA

Sequence:

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CAGGAAACGT TTCCTCCAAA GTACCTTCAT TATGACGAAG AAACCTCTCA TCAGCTGTTG 120
TGTGACAAAT GTCCTCCTGG TACCTACCTA AAACAACACT GTACAGCAAA GTGGAAGACC 180
GTGTGCGCCC CTTGCCCTGA CCACTACTAC ACAGACAGCT GGCACACCAG TGACGAGTGT 240
CTATACTGCA GCCCCGTGTG CAAGGAGCTG CAGTACGTCA AGCAGGAGTG CAATCGCACC 300
CACAACCGCG TGTGCGAATG CAAGGAAGGG CGCTACCTTG AGATAGAGTT CTGCTTGAAA 360
CATAGGAGCT GCCCTCCTGG ATTTGGAGTG GTGCAAGCTG GAACCCCAGA GCGAAATACA 420
GTTTGCAAAA GATGTCCAGA TGGGTTCTTC TCAAATGAGA CGTCATCTAA AGCACCCTGT 480
AGAAAACACA CAAATTGCAG TGTCTTTGGT CTCCTGCTAA CTCAGAAAGG AAATGCAACA 540
CACGACAACA TATGTTCCGG AAACAGTGAA TCAACTCAAA AATGTGGAAT AGATGTTACC 600
CTGTGTGAGG AGGCATTCTT CAGGTTTGCT GTTCCTACAA AGTTTACGCC TAACTGGCTT 660
AGTGTCTTGG TAGACAATTT GCCTGGCACC AAAGTAAACG CAGAGAGTGT AGAGAGGATA 720
AAACGGCAAC ACAGCTCACA AGAACAGACT TTCCAGCTGC TGAAGTTATG GAAACATCAA 780
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GTCACTCAGA GTCTAAAGAA GACCATCAGG TTCCTTCACA GCTTCACAAT GTACAAATTG 1140
TATCAGAAGT TATTTTTAGA AATGATAGGT AACCAGGTCC AATCAGTAAA AATAAGCTGC 1200
TTATAA

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[Brief Description of the Drawing]

[Figure 1]

It exhibits the result of Western blotting of the protein obtained by the expression of the genomic DNA of the present invention in example 3(iii)

[Explanation of Referenced Numerals]

1: marker

2: supernatant of culture medium of COS-7 cell transfected with vector pWESRαOCIF (example 3(iii))

3: supernatant of culture medium of COS-7 transfected with vector pWESR α (control)

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[Document] Abstract

[Abstract]

[Problems to be Solved]

A novel DNA encoding a protein having an inhibitory activity on osteoclast formation and a method of preparing said protein thereby.

[Means to Solve the Problems]

DNA described in Seq.ID.No.1 and 2.

A method of preparing a protein with a molecular weight of about 60 kD (under reducing conditions), and about 60 kD and about 120 kD (under non-reducing conditions) having an inhibitory action on osteoclast formation by inserting said DNA into expression vector and by genetic engineering manipulation. This protein has an inhibitory action on osteoclast formation and can be useful for curing osteoporosis and rheumatism.

[Selected Drawings] None

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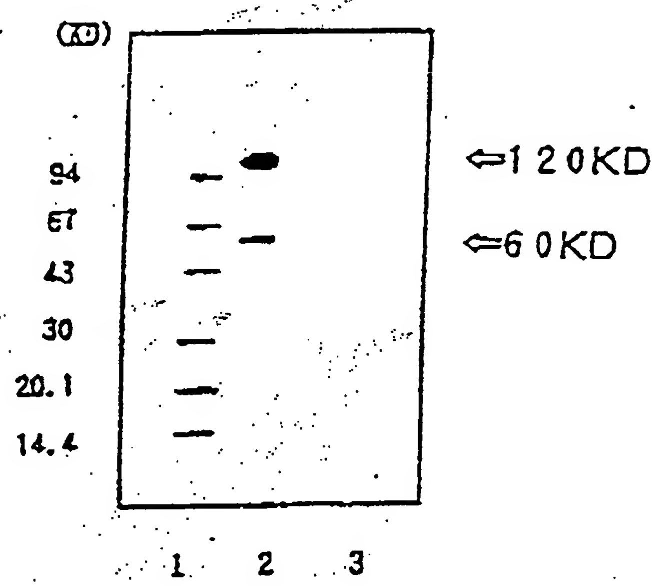
[Figure 1]



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CLAIMS:

1. A DNA comprising the nucleotide sequences of the Sequences No. 1 and No. 2 in the Sequence Table.

2. The DNA according to claim 1, wherein the Sequence ID No. 1 includes the first exon of the OCIF gene and the Sequence ID No. 2 includes the second, third, fourth, and fifth exons.

3. A protein exhibiting the activity of inhibiting differentiation and/or maturation of osteoclasts and having the following physicochemical characteristics,

(a) molecular weight (SDS-PAGE):

(i) Under reducing conditions: about 60 kD,

(ii) Under non-reducing conditions: about 60 kD and about 120 kD;

(b) amino acid sequence:

includes an amino acid sequence of the Sequence ID No. 3 in the Sequence Table,

(c) affinity:

exhibits affinity to a cation exchanger and heparin, and

(d) heat stability:

(i) the osteoclastogenesis-inhibitory activity is reduced when treated with heat at 70℃ for 10 minutes or at 56℃ for 30 minutes,

(ii) the osteoclastogenesis-inhibitory activity is lost when treated with heat at 90℃ for 10 minutes.

4. A process for producing a protein exhibiting an

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activity of inhibiting differentiation and/or maturation of osteoclasts and having the following physicochemical characteristics,

(a) molecular weight (SDS-PAGE):

(i) Under reducing conditions: about 60 kD,

(ii) Under non-reducing conditions: about 60 kD and about 120 kD;

(b) amino acid sequence:

includes an amino acid sequence of the Sequence ID No. 3 of the Sequence Table,

(c) affinity:

exhibits affinity to a cation exchanger and heparin, and

(d) heat stability:

(i) the osteoclastogenesis-inhibitory activity is reduced when treated with heat at 70℃ for 10 minutes or at 56℃ for 30 minutes,

(ii) the osteoclastogenesis-inhibitory activity is lost when treated with heat at 90℃ for 10 minutes,

the process comprising inserting a DNA including the nucleotide sequences of the sequences No. 1 and No. 2 in the Sequence Table into an expression vector, producing a vector capable of expressing a protein having the above-mentioned physicochemical characteristics and exhibiting the activity of inhibiting differentiation and/or maturation of osteoclasts, and producing this protein by a genetic engineering technique.

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5. The use of a DNA comprising the nucleotide sequences of the sequences No.1 and No.2 in the Sequence table, in the preparation of a medicament for the treatment of osteoporosis and rheumatism.

Snow Brand Milk Product Co. Ltd

By its attorneys

JAMES & WELLS

29 MAR 1999